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Rapid detection, identification, and enumeration of *Escherichia coli* by fluorescence in situ hybridization using an array scanner

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Abstract

A new fluorescence in situ hybridization (FISH) method using peptide nucleic acid (PNA) probes and an array scanner for rapid detection, identification, and enumeration of *Escherichia coli* is described. The test utilizes Cy3-labeled peptide nucleic acid (PNA) probes complementary to a specific 16S rRNA sequence of *E. coli*. Samples were filtered and incubated for 5 h, the membrane filters were then analyzed by fluorescence in situ hybridization and results were visualized with an array scanner. Results were provided as fluorescent spots representing *E. coli* microcolonies on the membrane filter surface. The number of fluorescent spots correlated to standard colony counts up to 100 colony-forming units per membrane filter. Above this level, better accuracy was obtained with PNA FISH due to the ability of the scanner to resolve neighboring microcolonies, which were not distinguishable as individual colonies once they were visible by eye. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fluorescence in situ hybridization (FISH); Peptide nucleic acid (PNA); *Escherichia coli*

1. Introduction

Bacterial contamination of foods, beverages, and water constitutes a serious threat to public health. Numerous examples of disease outbreaks due to contamination have been reported worldwide, and regulations for microbial limits have been issued. The reference method for detection and enumeration of bacterial contaminants often involves membrane filtration followed by growth on an appropriate medium and subsequent identification of colonies using morphological, biochemical, or molecular analyses.

Escherichia coli is part of the normal microflora of the intestinal tract of humans and warm-blooded animals and most strains are nonpathogenic. However, some serogroups, such as enterohemorrhagic O157:H7, are pathogenic and infection causes severe diarrhea and fever that can lead to death in young, old and immunocompromised hosts (Doyle et al., 1997). Several water and food borne outbreaks of enterotoxigenic *E. coli* have been reported (Bell et al., 1994; Center for Disease Control and Prevention, 1999). Additionally, *E. coli* is often monitored as an indicator of bacterial contaminants of fecal origin for quality assurance of municipal water and other prod-

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ucts for human consumption and health care (United States Pharmacopeia).

Methods for rapid detection and enumeration of microorganisms on membrane filters based on either ATP-bioluminescence (Millipore, Bedford, MA) or esterase activity (Chemunex, Maisons-Alfort, France) with complementary instrument platforms have been commercialized (Raynolds and Fricker, 1999; Upperman et al., 1999). These methods, however, do not provide identification. As a result, molecular and biochemical technologies have been developed to address the issue of both rapid and simultaneous detection, identification, and enumeration of microorganisms following membrane filtration. The biochemical methods utilize activities of cellular enzymes to detect individual cells (Van Poucke and Nelis, 2000a) or microcolonies following a short growth step (Sarhan and Foster, 1991; Bauters et al., 1999; Van Poucke and Nelis, 2000b). Direct fluorescence in situ hybridization (FISH) methods based on fluorescently labeled DNA probes (Muruyama and Sunamura, 2000; Tottorello and Reineke, 2000) and indirect FISH methods using biotinylated PNA probes (Prescott and Fricker, 1999) have been described for real-time analysis. However, the practical applicability of FISH has been limited to samples with a high level of contamination as the results are interpreted by manual epifluorescence microscopy allowing only a small part of a membrane filter to be viewed within a reasonable time. Recently, chemiluminescent in situ hybridization (CISH) using peroxidase-labeled PNA probes following a short-growth step have been described. This method detects individual CFUs as spots of light captured simultaneously by exposure to film (Perry-O'Keefe et al., 2001; Stender et al., 2000, 2001a).

Ribosomal RNA sequences are today widely accepted phylogenetic markers and have for the past decade been used to order and, in some cases, reorder the taxonomy of microorganisms (Fox et al., 1980; Kurtzman and Robnett, 1998). Differences between the highly conserved rRNA sequences of closely related microorganisms have enabled the use of species-specific rRNA target sequences for specific identification of microorganisms by molecular methods. The high cellular abundance of rRNA allows individual cells to be directly detected by fluorescence in situ hybridization (FISH) using fluorescence microscopy or flow cytometry (Delong et al., 1989; Amann et al., 1995).

Peptide nucleic acid (PNA) probes are DNA mimics with improved hybridization characteristics (Egholm et al., 1993; Nielsen et al., 1994), which have been exploited in variety of different molecular techniques (Thisted et al., 1996; Just et al., 1998; Taneja et al., 2001). Additionally, fluorescence in situ hybridization methods using fluorescently labeled PNA probes targeting rRNA (PNA FISH) for identification of microorganisms have been described (Stender et al., 1999, 2001b; Perry-O'Keefe et al., 2000).

Optical scanners based on either lasers or continuous light sources with appropriate excitation and emission filters are employed for the detection of hybridization reactions between fluorescently labeled oligonucleotides and combinatorial arrays of oligonucleotide libraries in a microscope slide format (microarrays). These scanners are designed to rapidly scan microarrays for the presence of fluorescent spots with high resolution. This microarray technology enables analysis of multiple gene expression patterns instead of single gene regulation, and is envisioned to accelerate the understanding of human diseases for future diagnosis and therapy; the next phase of the human genome project (Fodor, 1997). Furthermore, microarray scanners are being applied in the new areas of proteomics for high throughput functional analysis of protein–protein interactions (MacBeath and Schreiber, 2000).

In this study, we applied PNA FISH to membrane filtration following a short-growth step and utilized a high-resolution, microarray laser scanner to analyze entire membrane filters for fluorescent microcolonies of *E. coli*, hereby providing rapid and simultaneous detection, identification and enumeration of *E. coli* in filterable samples.

2. Materials and methods

2.1. Strains

Six *E. coli* strains and 17 other bacterial strains representing environmentally and clinically relevant bacterial species were obtained from various sources (Table 1). Unless otherwise noted, *E. coli*, ATCC

Table 1
Results of *E. coli* PNA FISH and culture following 5 h and overnight incubation, respectively

Organism	Strain	PNA CISH	Culture
Escherichia coli	ATCC #8739[a]	+	+
Escherichia coli	GMF/MSU[b]	+	+
Escherichia coli	WWTX[c]	+	+
Escherichia coli	MWTX[c]	+	+
Escherichia coli	ATCC #25922	+	+
Escherichia coli	MSC1[c]	+	+
Shigella sonnei	ATCC #29930	+	+
Shigella flexneri	ATCC #29903	+	+
Hafnie alvae	ATCC #13337	−	+
Pseudomonas putida	ATCC #12633	−	+
Serratia marcescens	ATCC #13880	−	+
Pseudomonas aeruginosa	ATCC #27853	−	+
Bacillus subtilis	ATCC #6633	−	+
Staphylococcus epidermidis	ATCC #14490	−	+
Staphylococcus aureus	ATCC #6538	−	+
Listeria innocua	ATCC #33090	−	+
Salmonella choleraesuis	ATCC #29946	−	+
Acinetobacter calcoaceticus	ATCC #23065	−	+
Citrobacter freundii	ATCC #8090	−	+
Enterobacter aerogenes	ATCC #49701	−	+
Klebsiella pneumonia	GMF/MSU[b]	−	+
Proteus mirabilis	ATCC #12453	−	+
Micrococcus luteus	ATCC #9341	−	+

+, Positive. −, Negative.
[a]American Type Culture Collection, Manassas, VA.
[b]Strain kindly provided by Montana State University, MT.
[c]Environmental isolate kindly provided by Millipore, Bedford, MA.

8739, was the representative strain for all experiments. The strains were propagated in either LB broth base (Sigma, St. Louis, MO) or TSB (Sigma or Difco Laboratories, Detroit, MI) at 30–35°C.

2.2. Sample preparation

Prior to filtration, strains were diluted in filter-sterilized PBS or 0.15 M NaCl. All samples were filtered through 25-mm PVDF membrane filters with a 0.45-μm pore size (Millipore).

2.3. Growth conditions

Membrane filters were aseptically transferred with forceps to Petri dishes containing TSA (Difco Laboratories) and incubated for 5 h at 35°C prior to PNA FISH analysis as described below. Visible colonies for standard colony counts were obtained following overnight incubation at 35°C.

2.4. Fluorescence in situ hybridization using PNA probes (PNA FISH)

The slide-based PNA FISH method described by Stender et al. (1999) was modified for membrane-based hybridization. Following incubation, microcolonies were fixed by placing membrane filters on pads soaked with 95% ethanol for 5 min at room temperature. The membrane filters were air-dried and then placed on microscope slides and covered with approximately 50 μl of hybridization solution containing 10% (w/v) dextran sulfate (Sigma), 10 mM NaCl (J.T. Baker), 30% (v/v) formamide (Sigma), 0.1% (w/v) sodium pyrophosphate (Sigma), 0.2% (w/v) polyvinylpyrrolidone (Sigma), 0.2% (w/v) ficoll (Sigma), 5 mM Na_2EDTA (Sigma), 0.1% (v/v) Triton X-100 (Aldrich), 50 mM Tris–HCl pH 7.5 and 100 nM Cy3-labeled PNA probe (TCAATGAGCAAAGGT) targeting *E. coli* 16S rRNA (Boston Probes, Bedford, MA). Coverslips were put on the membrane filters to ensure even coverage with hybridization solution, and the slides were subsequently placed in an incubator and incubated for 30 min at 50°C. Following hybridization, the coverslips were removed by submerging the slides into prewarmed 10 mM CAPSO, pH 10 (Sigma), 0.2% (v/v) Tween 20 (Sigma) in a water bath at 50°C, and the slides were washed for 30 min. The slides were, placed into individual slots in a PNA wash rack (Boston Probes) to keep the membrane filters separated. The membrane filters were then allowed to dry at room temperature and were finally mounted on microscope slides using one drop of immersion oil.

2.5. Laser scanning and image analysis

Membrane filters were scanned using a GMS 418 Array Scanner (Affymetrix, Woburn, MA) equipped with a 532-nm laser with the laser power and gain set at 20 and 19, respectively. The scanner was modified by the manufacturer to provide a resolution

of 5 μm. Images were captured as 16 bits tiff files and analyzed using Image Pro-Plus 3.0 (Media Cybernetics, Silver Spring, MD). Fluorescent microcolonies were determined as minimum 3 × 3 pixels with signals exceeding 1.5 times the membrane background and automatically counted by the software.

2.6. Fluorescence microscopy

Microscopic examination was conducted using a fluorescence microscope (Optiphot, Nikon, Tokyo, Japan) equipped with a 60 × /1.4-oil objective (Nikon), an HBO 100 W mercury lamp, and a Cy3 filter set (Omega, Brattleboro, VT). Images were obtained using a color CCD camera (Diagnostic Instruments, Sterling Hts., MI) connected to a computer system.

3. Results and discussion

3.1. Assay principle

The principle of this PNA FISH method in conjunction with an array scanner is illustrated in Fig. 1. *E. coli* was detected, identified and enumerated following 5 h of growth in the form of small spots of fluorescence representing individual microcolonies of *E. coli* on the membrane filter. In comparison, standard methods is based on sustained growth hereby generating visible colonies of both *E. coli* and *Pseudomonas aeruginosa* (i.e. detection and enumeration) and, therefore, require subsequent analysis for the final identification. Scanning was performed at 5-μm resolution, such that each pixel of the image corresponded to an area of 5 × 5 μm^2 of the membrane surface. Due to their very small size, not all microcolonies were clearly visible on the whole membrane

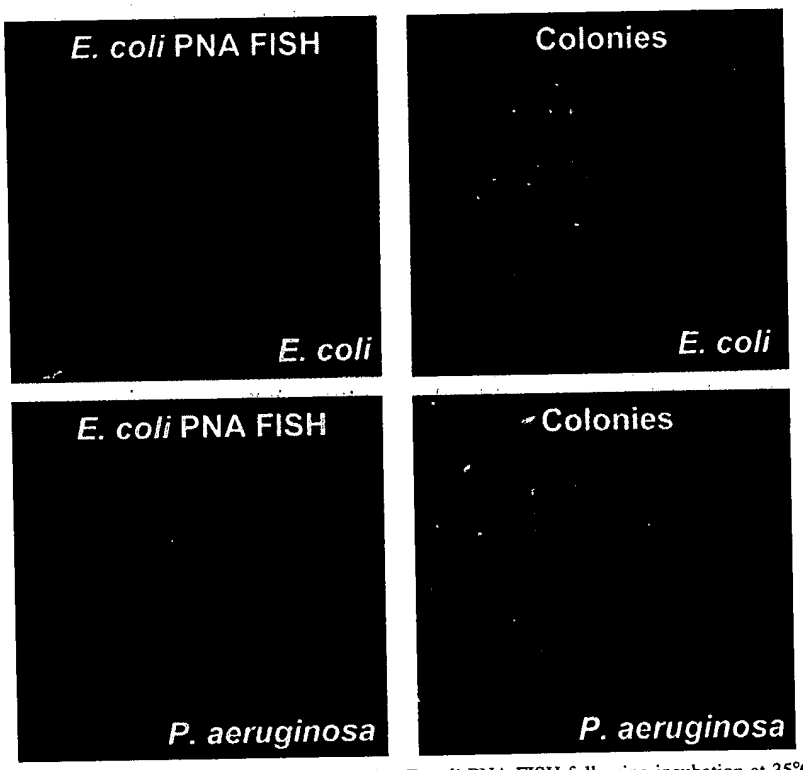

Fig. 1. Membrane filters with fluorescent microcolonies obtained by *E. coli* PNA FISH following incubation at 35°C for 5 h and duplicate membrane filters with visible colonies after overnight incubation from analysis of *E. coli* and *P. aeruginosa*.

image (Fig. 1). A close up of a laser scanner image along with a microscope image of the same microcolony showed that the microcolonies were spheroid with a diameter of approximately 50 μm (Fig. 2). The selectivity of *E. coli* PNA FISH was initially confirmed by the lack of fluorescent spots on the membrane with *P. aeruginosa* (Fig. 1). The selectivity of the laser scanner for the Cy3-labeled PNA probe was confirmed by the negative results obtained using a 635-nm laser with a corresponding emission filter for Cy5 (Fig. 2).

High-resolution scanning provided a high signal to background ratio that enabled direct detection of individual microcolonies without the use of enzymatic or other signal amplification technologies. Furthermore, high-resolution images allowed microcolonies to be visualized as a cluster of pixels rather than a single pixel without morphological characteristics. In this study, we used signal intensity and shape to determine a microcolony based on the digital image.

Each microcolony usually gave a fluorescent signal that was more than 10-fold greater than the background level at relatively low laser power (20%) and gain (19%). This is well above the cutoff set to 3×3 pixels with fluorescence intensities above 1.5 times the background and, therefore, allows for variability in the diameter and intensity of microcolonies. Even stronger signals may be obtained with higher laser power and gain, however, the inherent autofluorescence from the type of membrane filters used prevented a further increase of these parameters. It is, however, likely that membranes having less autofluorescence can be used or that the autofluorescence can reduced by the use of infrared fluorescent dyes. This would enable a further increase of the signal to background ratio that might result in a shortened growth step prior to analysis.

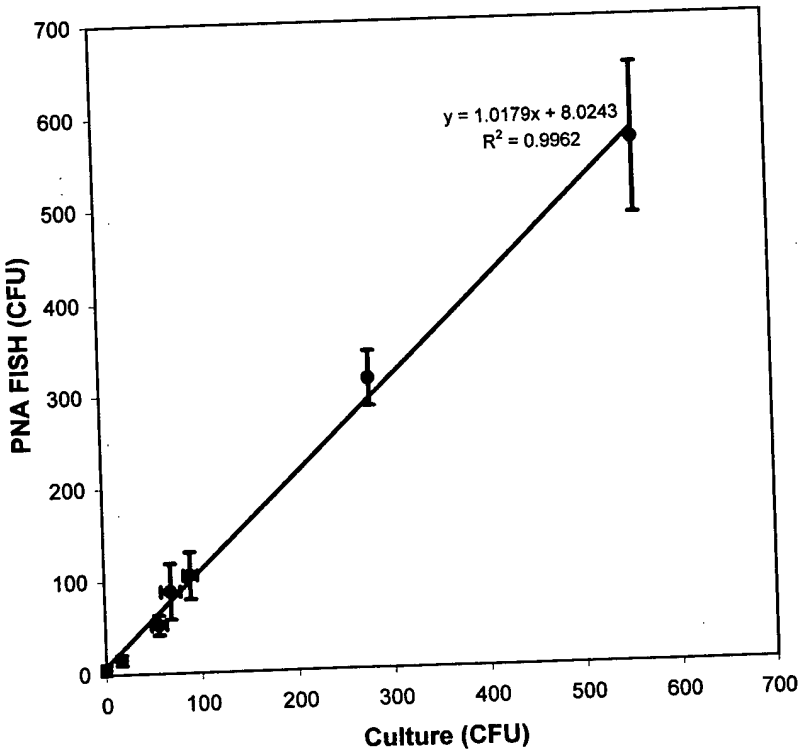

Fig. 3. Correlation between CFU per membrane filter determined by PNA FISH and standard culture method. Each data point is an average from analysis of three separate membrane filters for each dilution and error bars represent standard deviations. CFUs for standard culture method above 100 CFU are based on extrapolation of the average CFUs for dilutions with below 100 CFUs.

Fig. 2. Close up of one *E. coli* microcolony detected by *E. coli* PNA FISH. Images obtained with array scanner using laser for Cy3 (A) and Cy5 (B) as well as a corresponding microscopy image (C).

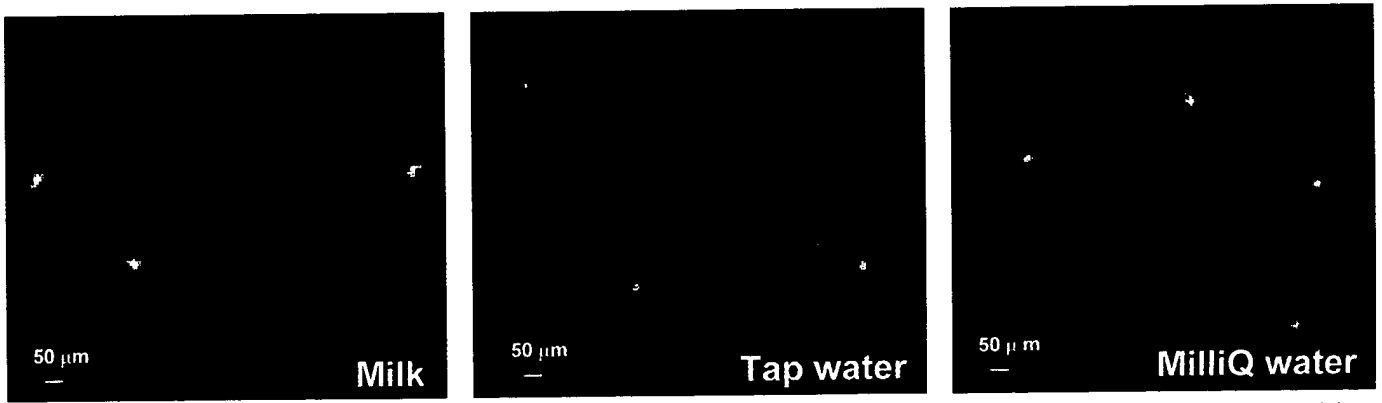

Fig. 4. Representative images obtained by *E. coli* PNA FISH from analysis of approximately 200 CFU of *E. coli* spiked into 50-μl milk (A), 100-ml tap water (B) and 100-ml MilliQ water (C).

3.2. Sensitivity and specificity of E. coli PNA FISH assay

Six *E. coli* strains and 17 other bacterium strains representing environmentally and clinically relevant bacteria were tested to assess the sensitivity and specificity of *E. coli* PNA FISH (Table 1). For all bacteria, visible colonies were obtained following overnight incubation. *E. coli* PNA FISH detected all of the *E. coli* strains as well as the two *Shigella* species whereas none of the remaining bacterial species were detected. Detection of the *Shigella* species was due to the very high degree of similarity between rRNA of *E. coli* and *Shigella* species such that a unique *E. coli*-specific target sequence does not exist (Kempf et al., 2000). This was in agreement with other applications using probes targeting rDNA or rRNA of *E. coli* (Tsen et al., 1998; Tottorello and Reineke, 2000; Stender et al., 2001a).

3.3. Correlation to culture

Data for the correlation between the number of fluorescent spots by PNA FISH and colony counts following overnight incubation are shown in Fig. 3. Above approximately 100 CFU, it became impossible to produce an accurate plate count because a lawn of overlapping colonies was produced. The use of PNA FISH and the laser scanner, however, allowed microcolonies to be resolved before they became confluent and, thus, provided a much broader dynamic range than standard plate counting. The slope of the linear regression line indicates approximately 100% correlation between CFU determined by *E. coli* PNA FISH and colony counts.

The microarray scanner has the potential of detecting a single microcolony on a membrane filter within 10 min. Considering that the membrane area of a 50-μm diameter microcolony is 1/250,000 of the total area of a 25-mm circular membrane, it becomes evident why manual microscope-based FISH methods have been applied only to membrane-based assays with relative high level of CFU (10^2–10^5 cells/membrane). The calculation also shows that approximately 10^5 50 μm microcolonies are required for a confluent layer of microcolonies on a 25-mm-diameter membrane filter. Each microcolony must be surrounded by pixels with background fluorescence in order to be counted as individual microcolonies. Combined with the calculations above it is estimated that up to 10^4 CFU per membrane filter can be accurately determined.

3.4. Applications

This rapid *E. coli* test may be applied to a variety of different applications within clinical, industrial and environmental microbiology. As examples, we spiked *E. coli* into 50-μl milk, 100-ml tap water, and 100-ml Milli Q water and analyzed those along with nonspiked samples by both *E. coli* PNA FISH and by prolonged incubation to form visible colonies. Examples of representative images obtained with *E. coli* PNA FISH of the spiked samples (Fig. 4) showed fluorescent *E. coli* microcolonies without background interference from the different sample matrixes. Overnight incubation of the nonspiked samples showed that the milk sample had a high level of indigenous bacteria, the tap water sample contained just a few other microorganisms, whereas the MilliQ water sample was sterile.

4. Conclusion

This new method combines membrane filtration for analysis of large sample volume with the sensitivity and specificity of PNA probes targeting rRNA and with the speed and high resolution of array scanners. Low levels of *E. coli* can, thus, be detected, identified, and enumerated following only 5 h of incubation as compared to current standard method based on overnight incubation followed by subsequent colony identification.

4.1. Multicolor multiplex applications

Multicolor multiplex PNA FISH using differently labeled PNA probes have been applied for simultaneous identification of four USP indicator organisms *E. coli*, *Staphylococcus aureus*, *Salmonella* spp., and *P. aeruginosa* using fluorescence microscopy (Perry-O'Keefe et al., 2000) and may be applied to this application using array scanners with multiple lasers. Although most scanners today are equipped with the lasers for Cy3 and Cy5, there is a trend towards

scanners being developed with additional lasers for a variety of fluorescent dyes. Additional multiplex applications may comprise the use of either universal dyes, such as 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide, biochemical reagents for universal enzymes, such as esterase or kingdom-specific PNA probes to obtain total counts combined with species-specific PNA probes for the detection of the presence of certain indicator organisms or pathogens.

4.2. The future

Array scanners are designed for expression analysis and genetic studies (i.e. genomics), a discipline that is currently experiencing a tremendous exploration (Gwynne and Page, 2000). Scanners are being commercialized by a variety of companies and additional features are constantly being developed. These include higher resolution, faster scanning, carousels for high-throughput testing as well as supplementary equipment for automation. Improvements that also will be of significant benefit to the application described here. Besides the many commercial available microarray scanners, other types of commercial available scanning instruments such as the ScanRDI (Chemunex) or the Laser Scanning Cytometer (CompuCyte, Cambridge, MA) may be applied to this membrane-based PNA FISH method. However, the price of these instruments currently exceeds 100,000 USD. Many of the current array scanners are less expensive, and it is very likely that the price of array scanners will be lower in the near future thereby making this rapid microbiological method attractive to a broader range of applications in various microbiological settings. We are currently evaluating the use of PNA FISH in conjunction with array scanners in this respect.

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